ORIGINAL ARTICLE

Nifedipine treatment reduces brain damage after transient focal ischemia, possibly through its antioxidative effects

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Stroke is a major cause of mortality and morbidity in hypertensive patients. This study investigated the effects of nifedipine, an L-type voltage-gated Ca^{2+} channel blocker, on ischemic lesion volume after focal cerebral ischemia and reperfusion in rats. Rats were subjected to 1 h of transient middle cerebral artery occlusion (MCAO). At 2 days after MCAO, the rats were randomized into two groups that were fed either a normal control diet ($n=10$) or a nifedipine (0.001%) containing diet ($n=11$) for 2 weeks. Nifedipine treatment significantly reduced ischemic lesion volume (116.5 ± 10.8 vs. $80.0 \pm 8.2 \text{ mm}^3$, $P<0.05$) without affecting body weight or blood pressure. It also decreased thiobarbituric-reactive substances, an index of lipid peroxide, (2.6 ± 0.4 vs. $1.7 \pm 0.1 \mu\text{mol g}^{-1}$ tissue, $P<0.05$) and increased glutathione peroxidase (54.9 ± 4.7 vs. $70.9 \pm 6.4 \text{ U g}^{-1}$ protein, $P<0.05$) and glutathione reductase activities (32.4 ± 1.4 vs. $39.9 \pm 2.7 \text{ U g}^{-1}$ protein, $P<0.05$) in the mitochondria from the ischemic hemispheres. These results suggest that nifedipine treatment can reduce ischemic lesion volume after focal cerebral ischemia, possibly because of the decrease in oxidative stress with an increase in antioxidant activities within the ischemic area.

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INTRODUCTION

Stroke is one of the major causes of mortality and morbidity in patients with hypertension. Calcium channel blockers (CCBs) have been widely used in the treatment of hypertension. A large scale clinical study, the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial, reported that CCBs could prevent stroke better than diuretics with a similar blood-pressure-lowering effect.¹

Oxidative stress has an important role in the pathogenesis of ischemic brain damage.^{2–4} During ischemia, a robust influx of calcium (Ca^{2+}) activates glutamate receptors that induce the generation of reactive oxygen species from mitochondria⁵ and reactive nitrogen species,⁶ leading to lipid peroxidation.⁷ L-type Ca^{2+} channels and N-methyl-D-aspartate receptors have a crucial role in the entry of Ca^{2+} into neurons.⁸ Thus, these results suggest that the oxidative stress associated with ischemic brain damage can be ameliorated by the blockade of Ca^{2+} entry via CCBs. In fact, previous experimental studies have demonstrated that CCBs could reduce stroke size and neurological deficits after focal brain ischemia.^{9–12} Lukic-Panin *et al.*¹⁰ demonstrated that CCBs reduced oxidative stress and infarct volume after transient middle cerebral artery occlusion (MCAO) in rats. These findings suggest that CCBs have favorable neuroprotective effects

against stroke. However, in previous studies, the treatment of experimental animals with CCBs was started before the onset of ischemia. The effects of CCBs on ischemic lesion size have not been examined with administration after the onset of cerebral ischemia.

Therefore, the aim of this study was to determine the effects of nifedipine treatment, when started after the ischemic insult, on focal brain ischemia induced by transient MCAO in rats. We also determined its effect on oxidative stress and antioxidant enzymes in the brain.

METHODS

Animal models

Male Wistar rats (6 weeks old) were purchased from KBT Oriental (Saga, Japan). The rats were housed in a temperature- and humidity-controlled room, and were fed a commercial diet (MF; Oriental Yeast Tokyo, Japan) and water *ad libitum*.

Focal cerebral ischemia was created in rats by transient MCAO as previously described.^{13,14} In brief, the rats were anesthetized with 2% isoflurane (Dainippon Pharmaceutical, Osaka, Japan) and maintained with 1% isoflurane using a face mask. A 1-cm midline incision was made on the anterior neck, and the right common carotid artery, external carotid artery and internal carotid artery were exposed. The common carotid artery and external carotid artery were

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ligated, and a suture was placed around the internal carotid artery for ligation. A thrombus was created by inserting a 4-0 nylon surgical thread into the internal carotid artery via a small incision. The middle cerebral artery was occluded by advancing the thrombus into the internal carotid artery to block the origin of the middle cerebral artery. After 1 h of MCAO, the middle cerebral artery was reperused by withdrawing the thrombus. During these procedures, the rectal temperature was maintained at 37 °C by placing the rat on a heating pad.

In our preliminary experiment, the changes in oxidative damage and antioxidant enzymes were assessed in the rat brain before the procedure ($n=6$) at 1 h after MCAO ($n=6$) and at 2 days after MCAO and reperfusion ($n=6$). To examine the effects of the CCBs on brain damage after transient focal ischemia, all surviving rats were randomly assigned to two groups at 2 days after MCAO, control diet ($n=18$, MF; Oriental Yeast) and 0.001% nifedipine-added MF diet ($n=18$, Oriental Yeast), for 2 weeks. The amount of nifedipine intake was estimated to be $\sim 0.8 \text{ mg kg}^{-1}$ body weight per day based on the amount of food intake in our animals. All procedures and animal care were approved by the Animal Care and Use Committee, Kyushu University, and carried out in accordance with the Guidelines for Animal Experiments, Kyushu University.

Animal characteristics

Arterial pressure and heart rate were analyzed by a Power Lab 4/30 with a blood pressure transducer (MLT0670, AD Instruments Japan, Nagoya, Japan). Arterial pH, pO_2 and pCO_2 levels were analyzed by a GASTAT-mini (Techno Medica, Yokohama, Japan).

Estimation of ischemic lesion volume by MRI

Ischemic lesion volume was estimated using a 0.4 T MRI (Hitachi Medical Corporation, Tokyo, Japan) and a standard T2-weighted fast spin echo sequence (Repetition time/Echo time/Flip angle, 3000 ms/90 ms/90-degree, for 2.5-mm-thick slices) as previously described.^{15–17} Briefly, after anesthesia was induced with pentobarbital (Dainabot, Tokyo, Japan), the rats were placed on a non-magnetic holder, which was placed into the bore of the MRI magnet. Spin-echo scout images were acquired first in the coronal and then in the sagittal planes to select reproducible coronal slice positions. A total of 12 contiguous coronal slice images were obtained from each rat. The ischemic lesions in T2-weighted imaging were defined as hyperintense areas on the image. To measure the ischemic lesion volume, the outline of the hyperintensive area was traced on the MRI, and then the sum of the areas gave the volume of the ischemic lesion volume in mm^3 .

Measurement of brain water content

Immediately after the rats were killed, the brain was removed, and the cerebrum was divided into ischemic and contralateral hemispheres. The wet weights of the tissue segments were measured. After segments were dried in a desiccating oven for 24 h at 100 °C, their dry weights were measured, and the percentage water content of the tissues was calculated.

Measurement of brain thiobarbituric-reactive substances (TBARS)

The concentration of TBARS was measured in both the ischemic and the contralateral hemispheres. After MRI experiments, the animals were killed by transcardiac perfusion with heparinized saline, and the brains were removed. The brain was homogenized (3:7, w/v) in 1.15% of KCl and 5 mM of 2,6-di-*t*-butyl-4-methylphenol. A 0.1-ml sample of the homogenate was mixed with reagents to give a final concentration of 2 mM EDTA, 7.5% acetic acid and 0.4% SDS and then reacted with 0.3% thiobarbituric acid (Wako Pure Chemical Industries, Osaka, Japan) in a boiling water bath for 45 min. After being cooled, the chromogen was extracted in *n*-butanol:pyridine (15:1, v/v). The TBARS concentration was calculated from the absorption values of the butanol-extracted supernatant at 532 nm. 1,1,3,3-Tetraethoxypropane was used as the standard.

Measurement of brain antioxidant enzymes

Mitochondrial and cytoplasmic fractions were isolated from both ischemic and contralateral hemispheres as previously described.¹⁴ Glutathione peroxidase

(GPx) and glutathione reductase (GR) activities in each fraction were measured according to the method of Yamamoto and Takahashi¹⁸ and Carlberg *et al.*,¹⁹ respectively. One unit of both enzyme activities was defined as the oxidation of 1 μmol of nicotinamide adenine dinucleotide phosphate per minute by measuring the absorbance measured at 340 nm. The superoxide dismutase (SOD) activity was determined by monitoring the inhibition of xanthine oxidase-mediated cytochrome *c* reduction from the absorbance at 550 nm.^{20,21} The specific activity was expressed as units per gram of protein.

Measurement of mitochondrial complex activities

Mitochondrial fractions were isolated from both ischemic and contralateral hemispheres. The specific activity of the mitochondrial complex enzymes, including complexes I, II, III and IV, was measured in the mitochondria isolated from the whole brains of each group as previously described.²² The enzymatic activities were expressed as units per gram of protein.

Statistical analysis

All of the results are shown as the mean $\pm$ s.e.m. Statistical significance was analyzed using the two-tailed *t*-test or Tukey–Kramer test. A probability value of 0.05 was set as the minimum level of statistical significance.

RESULTS

TBARS were significantly increased in the ischemic hemispheres compared with the contralateral hemispheres from rats at 2 days after MCAO and reperfusion (Figure 1). The GR activities in the mitochondrial fraction were significantly decreased in the ischemic hemispheres compared with the contralateral hemispheres from rats at 1 h after MCAO and at 2 days after MCAO and reperfusion (Figure 2a). The GPx and SOD activities in the mitochondrial fraction did not change in either group (Figure 2a). The GPx and GR activities in the cytoplasmic fraction from the ischemic hemisphere were significantly decreased in the ischemic hemispheres compared with the contralateral hemispheres from rats at 1 h after MCAO and at 2 days after MCAO and reperfusion (Figure 2b). The SOD activity tended to be lower in the cytoplasm from the ischemic hemispheres compared with the contralateral hemispheres from rats at 1 h MCAO and at 2 days after MCAO and reperfusion; this difference, however, did not reach statistical significance (Figure 2b).

There were no significant differences in body weight and food intake between control and nifedipine-treated rats. The mean arterial pressure, heart rate and blood gases were also comparable between the

Figure 1 TBARS in ischemic (closed bars) and contralateral hemispheres (open bars) from rats before the operation ($n=6$) at 1 h after MCAO ($n=6$) and at 2 days after MCAO and reperfusion ($n=6$). TBARS of the ischemic hemisphere were significantly increased in the rats at 2 days after MCAO and reperfusion. * $P<0.05$ vs. the contralateral hemisphere.

Figure 2 The GPx, GR and SOD activities in the mitochondrial (a) and cytoplasmic fractions (b) of the contralateral (open bars) and ischemic hemispheres (closed bars) from the rats before the operation ($n=6$) at 1 h after MCAO ($n=6$) and at 2 days after MCAO and reperfusion ($n=6$). * $P<0.05$ and *** $P<0.005$ vs. the contralateral hemisphere in each group of rats.

Table 1 Animal characteristics

	Control ($n=10$)	Nifedipine ($n=11$)
Body weight (g)	311.3 $\pm$ 14.9	292.6 $\pm$ 10.4
Food intake (g kg ⁻¹ body weight per day)	82.8 $\pm$ 4.7	78.9 $\pm$ 2.6
Mean arterial pressure (mm Hg)	97.4 $\pm$ 1.6	97.1 $\pm$ 2.3
Heart rate (b.p.m.)	395 $\pm$ 20	396 $\pm$ 11
pH	7.4 $\pm$ 0.02	7.4 $\pm$ 0.01
pO ₂ (mm Hg)	106.5 $\pm$ 7.4	107.5 $\pm$ 6.6
pCO ₂ (mm Hg)	41.5 $\pm$ 4.7	42.3 $\pm$ 5.5

Each value represents the mean $\pm$ s.e.m. The data were obtained at 2 weeks after treatment.

groups (Table 1), indicating that nifedipine did not exert significant hypotensive effects in this study.

Ischemic lesions were visualized by T2-weighted MRI. Ischemic lesion volume was significantly smaller in nifedipine-treated rats compared with control rats by 30% (Figures 3a and b). Water content was also lower in the ischemic hemisphere of nifedipine-treated rats compared with that of control rats (Figure 3c).

TBARS were significantly increased in the ischemic hemispheres compared with the levels in the contralateral hemispheres from control rats. TBARS in the ischemic hemispheres from nifedipine-treated rats were significantly lower compared with those from control rats and were comparable with those in the contralateral hemispheres (Figure 4).

The GPx and GR activities in the mitochondrial fraction were comparable between ischemic and contralateral hemispheres from control rats, whereas those in the mitochondrial fraction of the ischemic hemispheres were significantly increased compared with those in the contralateral hemispheres from nifedipine-treated rats (Figure 5a). The SOD activity tended to be lower in the mitochondrial fraction from the ischemic hemispheres compared with the contralateral hemispheres, but this difference did not reach statistical

significance (Figure 5a). The GPx activity in the cytoplasmic fraction from the ischemic hemispheres was significantly increased compared with that in the contralateral hemispheres of the control group, and this increase was similarly observed in the nifedipine-treated rats (Figure 5b). The GR and SOD activities in the cytoplasm did not change in either group (Figure 5b).

We measured the mitochondrial complex activities to estimate mitochondrial function, but these enzyme activities did not change in either group (Figure 6).

DISCUSSION

This study demonstrated that treatment with nifedipine, initiated even after the onset of ischemia, could reduce ischemic lesion volume after transient MCAO in association with the reduction in oxidative stress and the increase in mitochondrial GPx and GR activities in the ischemic brain.

Oxidative stress has an important role in the pathogenesis of ischemic brain damage.²⁻⁴ We have also demonstrated the accumulation of lipid peroxidation products and the decrease in antioxidative enzymes in the ischemic hemispheres in our model at 2 days after MCAO and reperfusion. Previous studies have already reported the direct neuroprotective effects of CCBs during the early phase of ischemia reperfusion.⁹⁻¹² This study has extended these findings and demonstrated for the first time that nifedipine can ameliorate brain damage even when administered after reperfusion. These findings might also suggest that the intracellular Ca²⁺ overload, which is probably because of the excessive Ca²⁺ entry through overactivated glutamate receptors, is a crucial event not only during the early phase of ischemia but also during the process of persistent brain damage. Neuronal Ca²⁺ overload may trigger the formation of intracellular reactive oxygen species.²³ Moreover, hydrogen peroxide has been shown to enhance the Ca²⁺ current through L-type Ca²⁺ channels in cultured rat dentate granule cells.²⁴ The vicious circle between ischemia, Ca²⁺ overload and oxidative stress may be involved in the ischemic brain damage, and cause further deterioration in the integ-

Figure 3 MRI images of ischemic lesion volume (a), summary data of ischemic lesion volume using MRI (b), and water content of the brains (c) in control and nifedipine-treated rats. Ischemic lesion volume and water content were significantly smaller in nifedipine-treated rats. # $P < 0.05$ vs. control rats. *** $P < 0.005$ vs. the contralateral hemisphere. ## $P < 0.01$ vs. the ischemic hemisphere from control rats.

Figure 4 TBARS in ischemic (closed bars) and contralateral hemispheres (open bars) from control ($n=10$) and nifedipine-treated rats ($n=11$). TBARS of the ischemic hemisphere was significantly reduced in the nifedipine-treated rats compared with control rats. *** $P < 0.005$ vs. the contralateral hemisphere. # $P < 0.05$ vs. the ischemic hemisphere from control rats.

rity of at-risk tissue. Therefore, CCBs may prevent these processes of Ca^{2+} overload and oxidative stress in the ischemic brain.

There is accumulating evidence that CCBs have antioxidative effects in the setting of lysophosphatidylcholine-induced injury,²⁵ salt-loaded, stroke-prone spontaneous hypertension,²⁶ atherosclerosis¹² and cerebral ischemia.^{10,27} It has also been reported to increase the activity of antioxidant enzymes, such as GPx, GR and SOD.^{21,27,28} In agreement with these previous results, this study showed that nifedipine treatment inhibited lipid peroxidation and increased GPx and GR activities in the mitochondria of ischemic brains (Figures 4 and 5). The increases of antioxidant enzyme activities in the mitochondria may decrease oxidative stress because the mitochondrial electron transport chain is one of the major sources of reactive oxygen species in ischemic brains.²⁹ In this study, SOD activities in the mitochondrial and cytoplasmic fractions of the brain were not altered (Figure 5). However, the results were not in agreement with previous studies in which SOD activity was increased by nifedipine treatment in the early phase of brain reperfusion.²⁷ In this study, nifedipine treatment did not affect blood pressure or heart rate, indicating that the neuroprotective effects of nifedipine are independent of its blood pressure-lowering effect on ischemic lesion volume.

Figure 5 GPx, GR and SOD activities in the mitochondrial (a) and cytoplasmic fractions (b) of the contralateral (open bars) and ischemic hemispheres (closed bars) from control ($n=10$) and nifedipine-treated rats ($n=11$). ** $P < 0.01$ and *** $P < 0.005$ vs. the contralateral hemisphere in each group of rats. # $P < 0.05$ vs. the ischemic hemisphere from control rats.

Figure 6 Mitochondrial complex activities in the contralateral (open bars) and ischemic hemispheres (closed bars) from control ($n=10$) and nifedipine-treated rats ($n=11$).

In this study, we estimated the ischemic lesion volume using T2-weighted MRI. Proton density in MRI indicates the density of the water proton spin; thus, the T1 and T2 of water proton spins depend on the physiochemical state of water. Many pathological changes, such as tumors, edema and hemorrhage, are associated with a change in water content. Therefore, T2-weighted MRI has been extensively applied to delineate lesions in both clinical and experimental diagnostic studies,^{30,31} because both T1 and T2 have been reported to correlate with tissue water content. In particular, the T2 change is shown to be larger than the T1 change.^{32,33} In fact, our data showed parallel changes in ischemic lesion volume and water content (Figure 3).

There are several limitations of this study. First, we did not measure cerebral blood flow in the ischemic and non-ischemic regions after MCAO. However, the rats were randomized after 2 days of MCAO. Therefore, the decrease in cerebral blood flow after MCAO may not differ between rats with or without nifedipine treatment. Second, we could not exclude the possibility that an increase of blood flow to the ischemic area via the improvement of endothelial function may be involved in the reduction of ischemic lesion volume in nifedipine-treated rats. Third, we have not examined whether the findings of this study can be extended to other CCBs, and we definitely need to perform additional studies to clarify this crucial issue. However, we feel that the beneficial effects of nifedipine may be observed with CCBs other than nifedipine because amlodipine and azelnidipine have been reported to inhibit apoptosis in an animal model of brain infarction due to 90 min MCAO and 24 h of reperfusion.¹⁰ Fourth, we have not examined whether the administration of direct antioxidants can exert similar beneficial effects on brain damage as nifedipine, which may support our findings about the mechanisms of action of nifedipine.

In conclusion, this study demonstrated that nifedipine treatment could reduce ischemic lesion volume and oxidative stress after MCAO in rats. These beneficial effects of nifedipine may be partly because of the antioxidative action via increasing antioxidative enzymes in the ischemic brain.

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